1969

A Study of the Germination Process of Seeds of Heteranthera Limosa.

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70-254

MARLER, James Earl, 1939-

A STUDY OF THE GERMINATION PROCESS OF SEEDS OF HETERANTHERA LIMOSA.

The Louisiana State University and Agricultural and Mechanical College, Ph.D., 1969
Botany

University Microfilms, Inc., Ann Arbor, Michigan

A Study of the Germination Process of Seeds of *Heteranthera limosa*.

A Dissertation

Submitted to the Graduate School of the
Louisiana State University
Agriculture and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Botany and Plant Pathology

by
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B.S., University of Miami, 1962
M.A., University of Texas, 1965
May, 1969

ACKNOWLEDGEMENT

The author wishes to express his gratitude to Dr. John B. Baker for his guidance, patience, and encouragement during these investigations and also during the preparation of this dissertation.

He also acknowledges the aid and encouragement of the members of his committee.

Special thanks go to Angela S. Blaize and Linda J. Rebowe for their excellent technical assistance.

The author extends special acknowledgement to his wife, Harriet, for her technical assistance in the preparation of this manuscript.

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ABSTRACT

The seed of the aquatic plant, Heteranthera limosa Willd., are seen to have two requirements for germination: anaerobic conditions and light. Dormancy involves the seed coat which acts as a mechanical barrier to germination.

The light requirement appears to be a phytochrome mediated system which is unique in that it is unaffected by anaerobic conditions.

The seed obtains its energy through fermentative respiration and seedling growth is unaffected by 10^{-2}M cyanide. Iodoacetate completely blocks germination and seedling growth.

Reducing agents are able to substitute for anaerobic condition if the O_2 concentration is below 10%. Reduced glutathione, cysteine, thiosulfate, mercaptoethanol, and mercaptoacetate were all capable of stimulating germination.

Histological studies show a progressive breakdown of intercellular material along a cleavage line of a trap-door through which the radicle emerges. The force required to open the weakened trap-door is provided by the expansion of certain aleurone grain containing cells of the embryo cotyledon.

INTRODUCTION

This dissertation originally began as a weed control study of an occasionally bothersome aquatic weed in rice, Heteranthera limosa. The seeds of this plant were field harvested and brought into the laboratory for seedling studies. However, it was immediately evident that the seeds would not germinate on moist filter paper at any temperature. When the seeds were placed on top of soil or sand and flooded with water, they germinated readily provided the water was not aerated. Further preliminary study indicated the seeds required an oxygen limited system and white light to germinate. A study was begun to determine the nature of dormancy involved and a possible physiological explanation of the process of germination. Dormancy can be imposed upon a seed by internal or external factors. Internal factors responsible for dormancy are: immaturity of the embryo, mechanical restraint of the seed coat, impermeability of the seed coat to water and/or gases, and the presence of an internal inhibitor. External factors consist of improper temperature and/or light conditions, external chemical inhibitors, insufficient water, and unfavorable atmospheric composition. A review of the literature revealed that this seed showed an unusual set of germination requirements. Though the

seed of Heteranthera responds to red/far-red light and oxygen tension, the mode of action of these two may be different than that reported for most other light responsive seeds. This dissertation will attempt an explanation of this unique type of germination.

LITERATURE REVIEW

The most interesting aspect of this dormancy study was the requirement for anaerobic conditions. The influence of atmospheric composition on seed germination is widely reported in the literature (7, 18, 52). In many cases the oxygen concentration directly influences germination either positively or, as in this case, negatively. The majority of seeds lose the ability to germinate as the oxygen tension decreases (6, 8, 16, 20, 64, 81, 86, 96, 104). Carbon dioxide has also been shown to influence the germination of certain seed (5, 6, 8).

Early workers noted that many seeds were capable of germination under water at or near anaerobic conditions (8, 17, 51, 57, 58, 82, 98). As early as 1905, Takahashi (95) showed that oxygen did not appear to be necessary for the germination of rice. Crocker and Davis (17) found that seeds of Alisma (water plantain) and Cynodon (bermuda grass) were capable of germination in atmospheres containing little or no oxygen. It was the work of Morinaga (62, 63), in 1926, which showed for certain seeds a definite requirement for anaerobic conditions for maximum germination. His study of 78 species indicated that forty-three were able to germinate under anaerobic conditions, and of these, two were stimulated by the lack of oxygen. He reported that Typha (cattail) and

Cynodon (bermuda grass) exhibited increasing germination as the oxygen tension decreased. Bohmer (11) later added a few species to the list that were more or less favored by reduced oxygen. Edwards (22) reported that Peltrandra virginica was able to germinate under pure hydrogen and in the total absence of oxygen. Siegel and Rosen (85) noted that the ability to germinate at low oxygen tensions was apparently widespread among the angiosperms. Though this phenomenon does not appear to be as widespread in the gymnosperms, Migita (58) found that Pinus densiflora was stimulated to germinate by low oxygen tensions.

The early studies on the effects of oxygen centered around the seed coat and the endosperm. The investigators were concerned with the problems of gas exchange and water uptake in seeds showing dormancy from the lack of oxygen rather than from the excess of it. The extensive work with Xanthium serves as an example (16, 20, 105). A large number of studies into the effect of the seed coat on gas exchange indicated that the seed coat structure was often highly suberized and was able to restrict the movement of gases in or out (7, 20, 28, 32, 38, 92, 106). As work progressed in this area the seed coat effect was often found to be more complex than just a restriction on water or gas movement. While

many seeds did show only a mechanical inhibition of germination which could be broken by scarification (9, 32, 36, 50, 62, 63, 88, 90), others combined a light and/or internal inhibitor with the mechanical effect on dormancy (2). Thus, these studies into the effect of the seed coat on germination branched out into three basic areas: light and temperature effects, chemical (inhibitor) effects and the mechanical effects of the seed coat on germination. It goes without saying, of course, that these three areas are interrelated.

Bohmer (11) noted that many of the seeds which he was testing for response to various oxygen tensions were sensitive to light. The classic paper by Flint and McAllister (25) established that the light effect showed an action spectrum of maximum stimulation by red (600 mμ) and inhibition of germination by blue (420 mμ) and far-red (750 mμ) light. Borthwick et al (12, 13) found that the action spectrum was similar to that seen for flowering and internode extension. They also found that this photoblastic response was freely reversible. Even though the qualitative aspect of the light action remained the same (red/far-red relationship), the quantity of light required for germination varied from seed to seed. Some seeds require a single illumination period while others require a definite photoperiod (10, 23). Downs (21), reported that for various species of the Bromeliaceae, the

seeds required repeated light periods for maximum stimulation of germination. Mittal and Mathur (61) using tomato seed, found that continuous white light inhibited germination about 45% compared to dark germination while a short day photoperiod stimulated a 40% increase in germination. The continuous light inhibition is thought to be due to a shift in the phytochrome$_{750}$/phytochrome$_{660}$ (P_{750}/P_{660}) ratio. This shift is due to the continuous far-red and blue light emitted by the light sources used. Negbi and Kóller (67) believed that two independent photoreceptors were involved in the lowering of the P_{750}/P_{660} ratio and the subsequent inhibition of germination. They postulated a separate pigment system for the absorption of high energy blue light (which worked in conjunction with the red/far-red absorbing phytochrome pigment) to inhibit germination. The existance of this new and separate pigment system is disputed by several workers who claim that phytochrome can also effectively absorb in the blue end of the spectrum (34).

The location of the light response probably varies with the species studied. Black and Wareing (9) indicated that the photoperiodic response was either centered in the seed coat or in the endosperm since excised embryos readily germinated in the dark as well as the light. Stearns and Olson (93) were of the opinion that in Tsuga, the light and temperature effect centered not

in the seed coat but in the endosperm. They also noted that the optimal photoperiod varied with temperature and that the stratification of the seeds for 10 weeks removed the low temperature and photoperiod requirement. According to the evidence presented by Sifton (88), Chen and Thimann (37), and Bachelard (3) it is very probable that the system responding to light during germination resides in the embryo. Honig (35) reported that the ability to respond to light was inherited both through the nucleus and the cytoplasma.

Vaartaja (102) determined that Betula responded to light only when given on a long day sequence. He found this response was also temperature dependent, being more pronounced at lower (10-15°) than at higher (20-30°) temperatures. It was soon evident that not only was red light stimulation of germination temperature sensitive, but so was the far-red inhibition (41, 56, 77, 80, 107). The extensive studies with lettuce seed serve to illustrate the complexity of the phenomenon. Hagen et al (31) saw the far-red inhibition of lettuce rise from 70% at 5° to 100% at 30°. Scheibe and Lang (80) theorized that the low temperature reversal of dormancy seen in lettuce was due to a temperature sensitive conversion of P_{750} to P_{660}. Low temperature slows this conversion and allows the germination process to proceed past a critical point.

High temperature (and far-red light) speed up the shift to P_{660} and germination is stopped. Ikuma and Thimann (41) concluded that red light induction was a physical effect and not temperature or oxygen sensitive. They showed that the pre-induction period (dark imbibition preceding light exposure) was the temperature sensitive period while the post-induction time was oxygen sensitive. Rath-Bejerana *et al* (77) could not agree with this idea that the cold and phytochrome sensitive systems were separate. They noted that cold treatment was able to reverse the far-red inhibition, and also that far-red at the end of the cold treatment was able to reduce germination by more than 50%. They believed that these temperature effects were linked to an increase in phytochrome responsiveness to red light.

Mancinelli and Tolkowsky (56) indicated that in cucumber at least, there was an actual increase in the phytochrome content in the dark or red light. This increase in the dark was temperature sensitive. They also reported that the effect of far-red was seen as a lowering of the phytochrome content and the inhibition of germination. Yaniv and his coworkers (107) advanced the idea that in tomato and cucumber, temperature was involved in the rate of synthesis of active P_{750} and its reconversion in the dark to the inactive P_{660}.

Paralleling and often integrated with much of the work on the effect of light and temperature on dormancy is the use of various chemical agents to induce germination (9, 18, 52, 69, 76, 105). Various inorganic compounds have been seen to induce germination to some extent (23, 94). The most effective of these are $KMnO_4$, KNO_3, $MgSO_4$, H_2O_2.

The use of certain organic compounds to induce germination has open a new area of seed research. Thompson and Kosar (97), in 1939, surveyed a variety of sulfur compounds and reported that thiourea was able to replace the light and temperature requirement in lettuce. Poljakoff-Mayber and Evenari (72) found that the thiourea effect appeared to shift the respiration system from a fermentative to an oxidative system. They also noted that thiourea strongly inhibited the ascorbic acid oxidase enzyme. This thiourea stimulation is not universal, however, and Roberts (76) found that rice was not affected by it. Hashimoto (33) also noted a lack of response to thiourea by Nicotiana tabacum.

Ruge (79) reported the soaking of seed in glutathione tended to increase the rate of germination. Pollack and Kirsop (73) found the same effect in the germination of barley. However, Roberts (76) noted that with rice, sulfhydryl compounds had no stimulatory effect on germination.

It has been postulated by Rowsell and Goad (78), and Pollack and Kirsop (73) that the action of various sulfhydryl compounds is the release of bound enzymes required for germination. Rowsell and Goad (78) found that amylase activity in barley increased markedly following treatment with cysteine or reduced glutathione. Albaum et al (1), using oats, reported that oxidative conditions interfered with the proteolytic breakdown in the endosperm and ultimately resulted in the blockage of germination. Siegel and Porto (84) believe the growth and development of embryo during germination depends on a balance between oxidants and anti-oxidants. Reducing or anaerobic preconditioning often enhances the development of the embryo while elevated oxygen tensions are sometimes seen to be toxic to seeds and other plant tissue (83).

In the general area of growth regulating compounds, auxin (IAA) has been used with only limited success in stimulating germination. Only a few species have been reported to respond to auxin treatment (53). The most extensive work in this area has been on the action of gibberellin and kinetin on seed germination. Kahn and Goss (46) first reported, in 1957, that gibberellin was able to substitute for red light in the germination of lettuce seed. A general survey by Kallio and Puroinen (45) showed a wide variety of seeds were stimulated by

gibberellin treatment. They noted that several seeds that had shown negative results would respond when the seed coats were treated with acid before gibberellin treatment. Kahn (47) reported a close parallel between the action of red light and gibberellin promotion of germination in lettuce; however, he found that far-red light could not reverse gibberellin induced germination. He noted that even though there was some evidence for separate modes of action, gibberellin increased the responsiveness of the seeds to red light. Extensive studies by Ikuma and Thimann (37, 38) seemed to indicate a separate mode of action for gibberellin from that of red light. They reasoned that since red light does not produce gibberellin-like compounds in the seed, and that far-red light does not block gibberellin induced germination, the effect of gibberellin is upon a different system. Mittal and Mathur (61) noted that light effects and gibberellin effects were additive but they believed that these two systems were separated because of the non-reversal of gibberellin induced germination by far-red light. Ikuma and Thimann (37) advanced the theory that the action of gibberellin is similar to red light in that it activates (or stimulates some process to activate) hydrolytic enzmes to attack the seed coat. Bachelard (3) also concluded from his investigation of the action

of gibberellin on Eucalyptus that there was an enzymatic breakdown of cell wall material during germination. Ram-Chandra and Varner (75) showed that gibberellin action on barley involved RNA synthesis and believed that the proteins produced were enzymes needed for germination.

Simpson and his coworkers, Naylor, (65, 89) investigated the relationship between gibberellic acid, maltase, and amylase in the germination of barley. They concluded that germination required an accumulation of sugars and dormancy was due to the presence of an inhibitor which interfered with this accumulation. They observed that both exogenous sugar and gibberellin overcame the dormancy, and the role of gibberellin was to effect the release of the enzyme maltase which is thought to be found up in an inactive form in the endosperm. The current interpretation is that the enzyme is bound by disulfide (-S-S-) bonds and gibberellin somehow effects the reduction of the bonds. Roberts (76) reported that in rice, gibberellin stimulated germination but various reducing agents had no effect on germination. Paleg (70) observed that α-amylase activity in barley was directly influenced by gibberellic acid. Varner (103) found this enzyme to originate from the aleurone layers. He noted that chloramphenicol inhibits amylase production, and further work showed that most of the protein produced by

these cells was in the form of amylase enzyme. It was shown that RNA synthesis must occur before the aleurone cells would respond to added gibberellic acid, and it was not certain at what stage in enzyme production gibberllin was exerting its influence.

Scheibe and Lang (80) reported that gibberellin treatment appeared to overcome an inhibition of water uptake by the embryo in lettuce seed. The evidence presented indicated that red light or gibberellin was acting on the embryo, allowing osmotically active compounds to be released, and the subsequent water uptake led to elongation of the radicle, i.e. germination. Chen and Thimann (15), working with the light-inhibited seed of Phacelia, also supported the idea of osmotic control by light and gibberellin. Light blocked the release of osmotically active material, while gibberellin overcame this inhibition, allowing the osmotic uptake of water by the seed and its subsequent germination. They could find no increase in starch-hydorlyzing enzymes nor could they report any evidence of seed coat breakdown. They did note that actinomycin-D blocked gibberellin-induced germination and concluded that some type of enzymatic action may be responsible for the release of osmotically active molecules.

The presence of internal gibberellin-like compounds

has been detected in various seeds. Curtis and Cantlon (19) studied the presence of a gibberellin-like compound in Melampyrum and noted the decrease of this compound as dormancy set in. Naylor and Simpson (65) and Franklin and Wareing (26) reported that as Avena and other seeds came out of dormancy there was an increase in internal gibberellin-like compounds. Lang (53) offers an extensive list of seeds containing this compound in his review of inhibitors and stimulators of germination; however, the exact role of these compounds is not well understood. Often there compounds may be found in high amounts in the seed and yet these seed remain dormant. There are considerable conflicting data in the literature concerning the presence and role of these compounds in seeds.

The use of cytokinins for the stimulation of germination began with the work of Miller (59) in 1956. He found that kinetin appeared to replace red light in the induction of germination in lettuce seed. It was also noted, however, that far-red light did not reverse this induction. Later he reversed himself and reported that kinetin was not able to replace red light. He found that stray light had leaked into his system and he concluded that only a synergistic effect was seen with light and kinetin (60). Haber and Tolbert (29) gave evidence to indicate that dark germination did indeed occur, and

there was not synergistic action. Skinner et al (91) observed a synergistic action of kinetin and other 6-substituted purines with gibberellic acid. They believe that these two classes of compounds may be intermediates in a common pathway. Ikuma and Thimann (40) compared the action of kinetin to that of gibberellic acid in lettuce. They observed only a slight stimulation of germination with kinetin in the dark but saw a synergistic effect by red light or gibberellin with kinetin. They proposed that the mode of action of kinetin was the promotion of expansion of the cotyledons. The role of red light and gibberellin was centered in the axis and thought to be the triggering system that allowed kinetin to exert its action.

Kahn (48) induced germination of the upper seed of Xanthium with low concentrations of kinetin and red light. He further advanced the idea of Tuan and Bonner (100) that dormancy was associated with the repression of gene expression. Kinetin and red light appeared to affect DNA dependent RNA synthesis selectively with only the synthesis of certain proteins being affected, not general protein synthesis. This parallels the work by Ram-Chandra and Varner (75) on gibberellic acid effects on RNA synthesis.

Leff (54) failed to see any dark stimulation of

lettuce seed with kinetin. She observed an interaction between kinetin and the ability of lettuce seed to respond to light. When the sensitivity to light was lost, the seeds failed to respond to kinetin treatment. It appeared to her that there was interaction between a late product of the photoreaction and some form of the kinetin.

Kahn (49) investigated the reversal of abscisic acid inhibition of germination of lettuce by kinetin. Abscisic acid blocked the synthesis of amylase and also coleoptile growth. Kinetin treatment resulted in reversal of this inhibition. When it was seen that abscisic acid blocked red light and gibberellin induced germination, kinetin was found to effectively reverse this. Normally kinetin would have little effect on any of these processes. Therefore, Kahn suggested that red light (and gibberellin) act at totally different sites in the germinative process than kinetin and abscisic acid. Red light and gibberellin appear to be the primary stimulus for germination and kinetin acts at a distant part of the pathway, or in the case of abscisic acid inhibition, at the sites of inhibition by abscisic acid on the germinative process.

METHODS AND MATERIALS

General

Experimental organism

Heteranthera limosa Willd., a monocot and a member of the Pontederiaceae family, is a common aquatic weed in rice fields of south Louisiana.

Plant propagation and seed production

Seeds of this plant were collected in the summer of 1967 and planted in 7"x11"x4" plastic trays slightly embedded in 2" of moist soil. The trays were flooded with 2" of water. Germination took place in about 3 days and the trays were transferred to growth chambers. Standard growth conditions consisted of a constant 30°C and a photoperiod of 16 hours light and 8 hours dark.

The first flower emerged in about 2-3 weeks and the flowers were hand pollinated as they appeared each day. From pollination to seed release took 14 days and the seed pods were harvested just prior to seed release. The seeds were stripped from the pods, air dried, and stored in the dark at room temperature (25-30°). Seeds to be used in the experiments were washed in distilled water until thoroughly wet, surface sterilized by soaking in a 2% sodium hypochlorite solution for 5 minutes and then thoroughly washed

with sterile distilled water. The seeds were vacuum dried to remove surface moisture and stored in sterile screw cap vials. The seeds were kept in the dark since they tended to darken and lose their ability to respond to light if stored in strong white or fluorescent light. In the dark, the seeds retain their ability to germinate for at least two years (the duration of this study).

The seed of H. limosa is barrel shaped with a circular trap door at one end (Figure 8). The seed averages 0.7 mm long by 0.3 mm wide. It is brown in color and the color deepens if left in strong light. There are about 200-300 seeds per pod and they average 15 seeds per milligram dry weight or about 70 µg dry weight per seed.

Chemicals and Gases

All chemicals used were reagent-grade quality or the highest quality commercially available. The abbreviations or common names for certain chemicals used are as follows: 3-indoleacetic acid, IAA; dinitrophenol, DNP; furfuryl adenine, kinetin; gibberellic acid, gibberellin.

Germination studies under various atmospheres were performed using the highest purity of commercially available H_2, N_2, He, Ar, O_2, and CO_2 gases.

Standard Germination Conditions and Procedures

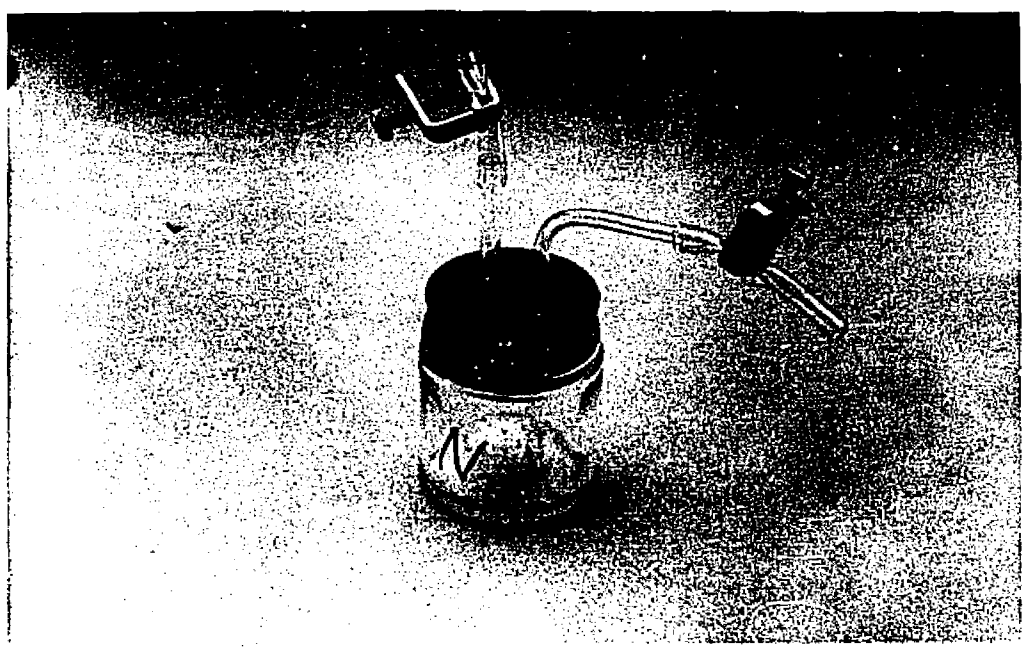
In the following experiments, the standard apparatus for germination consisted of a 100 ml specimen jar fitted with a size 10½ rubber stopper (Figure 1). The rubber stopper had two glass tubes inserted to allow gas to flow in and out of the jar. The tygon tubing on the ends of the glass tubes was fitted with Hoffman tubing clamps to seal the jar after gassing. It was found that commercial nitrogen flowing at 65 l/min (or 650 flask volumes/min) for 60 seconds was sufficient to flush the air out of the jar and allow germination to occur on moist 5 cm Whatman No. 1 filter pads. A light intensity of 1800 ft-c was provided by a combination of fluorescent and tungsten bulbs in constant temperature growth chambers. The standard germination conditions consisted of: N_2 atmosphere, 30°, and 1800 ft-c light given in a 16-8 (hours) photoperiod. Germination normally occurred in 72-84 hours. Each experiment contained an average of 30 seeds per germination flask and was run in duplicate or triplicate series per treatment. Figures cited in graphs or tables are an average of two or more experiments.

Respiration and Inhibitor Experiments

Respiration studies were performed using a Bronwill Warburg apparatus, model UVL (101). The studies (24-72

Figure 1. The vessel used for the germination of seed of H. limosa.

The germination vessel consists of a 100 ml specimen jar, no. 10½ rubber stopper, inlet and outlet tubes, and two Hoffman tubing clamps. The seeds can be seen as small black spots on the filter paper in the jars.



hours in duration) were carried out under sterile conditions to minimized bacterial growth. The seeds (30 mg) were weighed out and placed on sterile filter paper (Whatman No. 42) cut to fit the floor of the vessels. The filter paper was wet with sterile distilled water (0.4 ml). The vessels were flushed with nitrogen gas for 3 minutes to remove all oxygen. One-tenth milliliter of inhibitor or water was placed in each side arm and tipped in at the appropriate time.

Embryo Isolation Experiments

The isolation of embryos was accomplished through the use of finely sharpened needles and forceps. Isolation was performed under aspectic conditions. The embryos were extracted by splitting the seed coat lengthwise and carefully lifting them out of the endosperm tissue. The extraction was performed in a dark room using a safelight of green and yellow cellophane wrapped twice around a 15 watt G.E. no. F15T8-G6 Photo-Green fluorescent bulb (68). This light had no stimulatory effect on seed germination. After removal from the seed, the embryos were placed on a modified White's medium (55) containing 2% sucrose and 1% agar. The incubation period was five days at 30°C in light or dark. Growth was recorded as elongation of the cotyledon and as differentiation of the root system.

Oxygen and Nitrogen Mixture

The oxygen/nitrogen mixtures were made by filling a previously nitrogen-flushed low pressure tank with 10 lb/in^2 of nitrogen and adding the required lb/in^2 oxygen, after which, nitrogen was added up to the 50 lb/in^2 level to give the required percent oxygen in nitrogen mixture.

Light Response Study

Light quality experiments

Light quality survey experiments were conducted by wrapping the germination flasks with Dupont cellophase (Dennison Manufacturing Co., Farmingham, Mass.). The spectral qualities of the cellophane were determined using a Perkin-Elmer spectrophotomer, model 202. Maximum light transmission for each cellophane filter was as follows: red-650 mμ, green-540 mμ, blue-420 mμ, and far-red-750 mμ (Figure 2). A green and red cellophane combination was used to produce the far-red source. A combination of fluorescent and tungsten lamps were employed for the illumination source.

Red/far-red experiments

Detailed red/far-red studies were undertaken using a modification of the apparatus described by Poff and Norris (71). This apparatus was arranged vertically and the germination jar was placed horizontally in a light

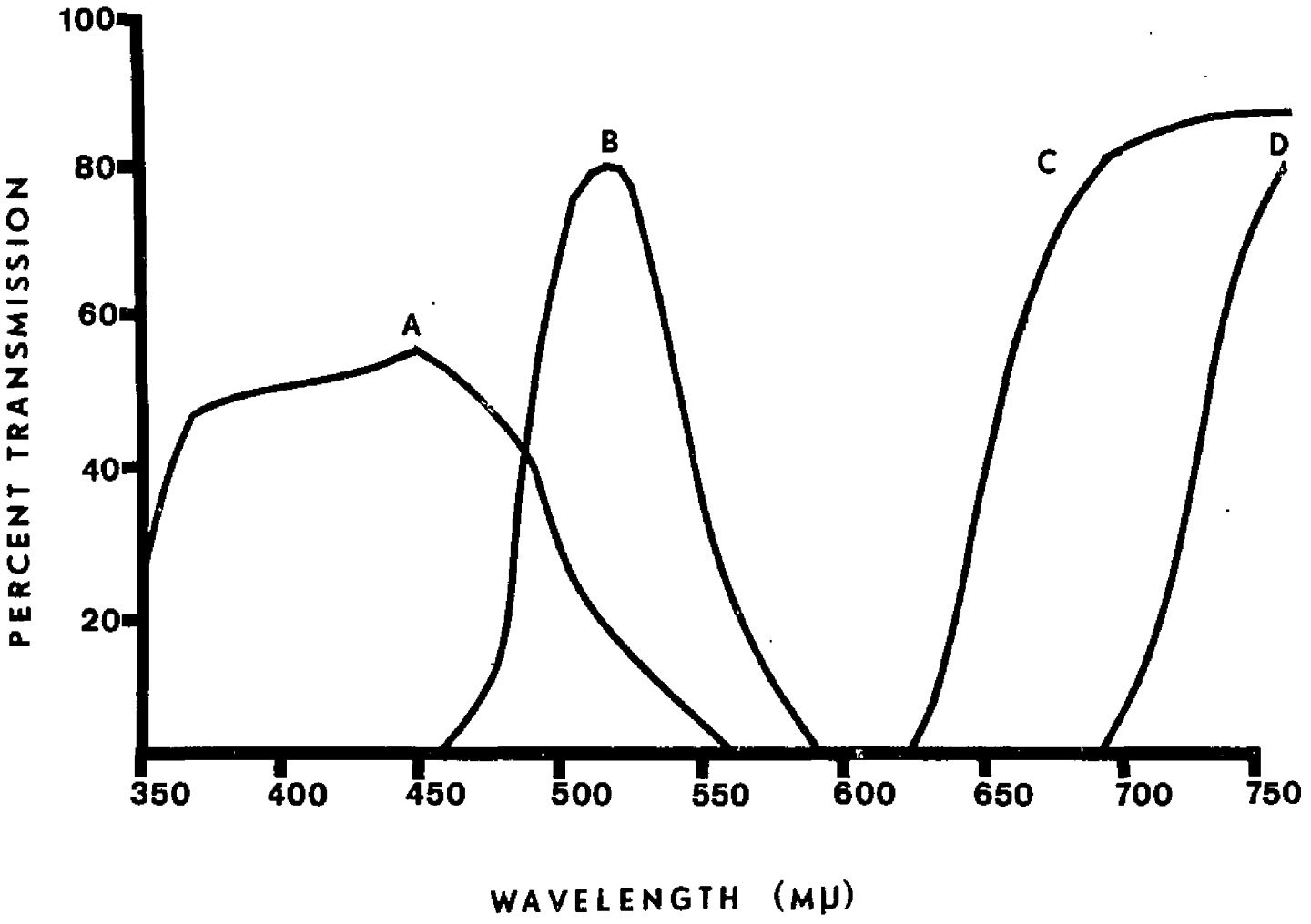
Figure 2. Transmission spectra of various cellophane filters.

A. 4 layers of blue cellophane.

B. 2 layers of green cellophane.

C. 2 layers of red cellophane.

D. 2 layers of green and 2 layers of red cellophane.


100
80
60
40
20
PERCENT TRANSMISSION
A
B
C
D
350
400
450
500
550
600
650
700
750
WAVELENGTH (Mμ)

proof box at a distance of 70 cm (for the far-red) and 77 cm (for the red) from the light source. The light was furnished by a 150 watt reflector flood lamp. The red and far-red filters were purchased from the Carolina Biological Supply Co., number CBS Red 650 (size 4"x4") and number CBS Far-red 750 (size 4"x4") respectively. The red and far-red filters were used as received from the company, and not combined as described by Poff and Norris. The red light induction consisted of three 8 hour illumination periods separated from each other by one hour of dark. The one hour of dark allowed the cooling of the light induction apparatus which tended to overheat under continuous light treatment.

Histological Study

Fixing, embedding, sectioning

Histological studies were conducted by the standard procedures outlined in detail by Jensen (43) and Johansen (44). The seeds were killed and fixed in an alcohol: acetic acid: formalin (90:3:7 by volume) solution after the seed coat had been slightly cracked to facilitate the penetration of the reagents. Dehydration was performed by an ethyl alcohol to tertiary-butyl alcohol series and the seed were embedded in 'Paraplast' (Aloe Scientific, St. Louis, Mo.). The seeds were sectioned on a Spencer rotary microtome, model 815.

Attempts were made to embed the seeds in a 'plastic' as recommended by Feder and O'Brien (24). Maraglas epoxy embedding medium (Polysciences, Inc., Rydal, Pa.) was prepared according to instructions furnished and the seeds were embedded. The standardized medium formulation was found to be the best of the seven formulations given. A vacuum oven was used to remove the numerous air bubbles formed during the filling of the embedding capsules. It was found that sectioning with the standard microtome was possible and gave excellent sections of the seed coat, as compared to the paraffin embedded sections of the seed coat. No difficulty was experienced in using the stains normally used in paraffin-section staining.

Stains and staining procedure

All stains and procedures were those given in Jensen (43) or Johansen (44). Total protein staining was by the ninhydrin-Schiffs reaction as outlined by Jensen. Lignified and suberized tissues were differentially stained with light green/sudan IV/hemalum or methylene blue/ruthenium red. The Feulgen nucleal reaction (counter stain with fast green) was used for the determination of chromosome division. General differential staining was done with safranin, counter stained with fast green. Starch was detected using the I_2/KI reagent.

Phase-Contrast Microscope Study

Phase-contrast studies were performed using a Bausch and Lomb phase microscope. The embryos were mounted in glycerine, covered with a cover slip, and squashed gently to release the aleurone grains. The grains were suspended on a cover slip in a "hanging drop" over one cavity of a dual cavity hanging drop slide. A drop of distilled water was placed in the other cavity. A ridge of paraffin around the edge of the cover slip allowed water vapor to reach the grains. The effect of water on the grains was viewed with dark field phase contrast microscope.

RESULTS

Studies on the Effect of Oxygen on Germination

Initial attempts to germinate fresh seed of *Heteranthera limosa* on moist filter paper failed. However, it was noted that the seed readily germinated when placed on moist soil or sand and covered with two inches of nonaerated water. The seed also germinated in flasks of water through which N_2 was bubbling, whereas, aeration with air prevented germination. It became apparent that these seed required an anaerobic environment for germination.

After experimenting with various germinating chambers, the jar shown in Figure 1 was determined to be the most satisfactory. This jar allowed the seeds to be placed on a 5 cm filter paper pad which could easily be lifted out of the jar for counting or examination.

To determine the effect of various gases on germination, N_2, H_2, He, Ar, and CO_2 were used to flush the jars. From the results of Table 1, it was seen that germination depended on the removal of oxygen rather than the addition of a specific gas. Air is primarily N_2 and O_2, and since germination could proceed normally in N_2, oxygen was likely the factor responsible for the inhibition of germination. Many seeds are able to germinate under extremely low O_2 concentration but most do require a trace of oxygen. To test this seed's

ability to germinate under zero oxygen tension, oxygen was completely removed with alkaline pyrogallol, and as seen in Table 1, the seed readily germinated. However, root and shoot development were retarded after germination occurred. The introduction of a trace of O_2 into the jar reversed this retardation.

The maximum amount of oxygen that the seeds could tolerate was somewhat more than 0.5%. Germination was inhibited about 80% at oxygen levels of 7.6 mm Hg or 1%, and inhibited 100% at oxygen levels above 1% (Figure 3).

Studies on the Light Requirement for Germination

Early in the atmospheric study, another germination requirement became apparent, the need for light during germination. Germination occurred in continuous light as well as an 8 hour day, but it was interesting to note that the germination time varied with the photoperiods shown in Table 2. This indicated a quantitative nature to the light requirement. It also took three times as long to germinate when the light intensity was cut by one-third. Continuous high intensity light (1800 ft-c) stimulated germination in 3 days while continuous low intensity light (600 ft-c) resulted in a 10-12 day germination time. When the light intensity times time product was equal, as comparing the continuous 600 ft-c light with the 8 hour photoperiod in high light intensity the germination time was also equal (Table 2).

Table 1. The influence of various gases on the germination of seed of H. limosa.

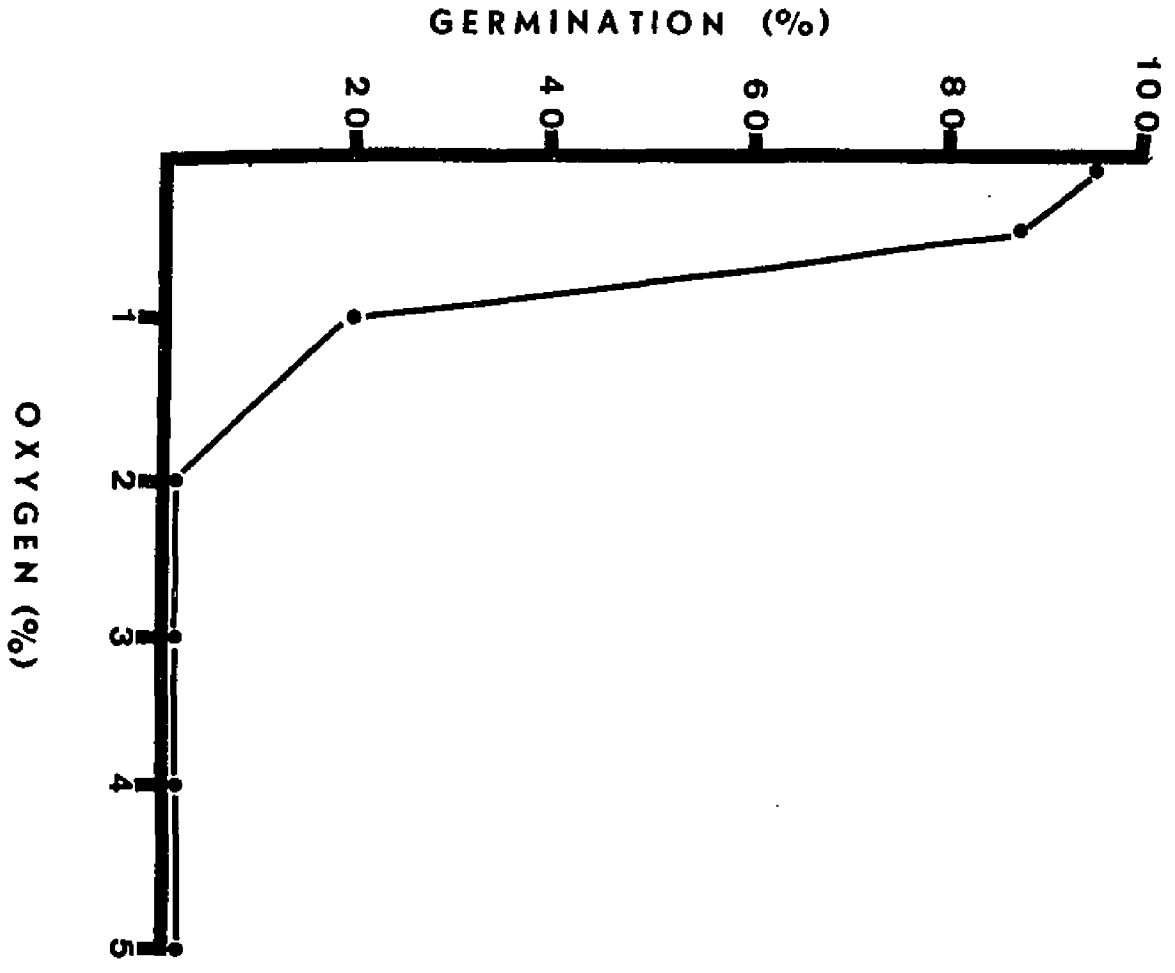
Gas	Percent Germination
Air	0
H_2	95
N_2	96
He	93
Ar	97
N_2 + pyrogallol	89*
CO_2	58

* Germination was recorded as the emergence of the radicle. The seedlings did not develop in the pyrogallol treated atmosphere.

Table 2. The effect of photoperiod and relative light intensity on germination and the time required for maximum germination.

Photoperiod Day	Night	Light Intensity	Ft-c hrs X 1000	Percent Germination	Germination Time (days)
24	0	1800 ft-c	129	90	3
16	8	" "	86	91	3
12	12	" "	108	90	5
8	16	" "	144	88	10
24	0	600 ft-c	144	89	10-12

Figure 3. The effect of various concentrations of oxygen (in N_2) on the germination of H. limosa seeds. Percent germination was determined after five days under standard conditions.


GERMINATION (%)
20
40
60
80
100
OXYGEN (%)
1
2
3
4
5

This seed required a prolonged treatment of red light for germination. An illumination period of 24 hours of red light was required to stimulate germination. The red light induction could be given either as continuous or as a total of 24 hours of light, in that three 8 hour light periods separated by 1 hour darkness was as effective as continuous light (Table 3). Three hours of far-red light was sufficient to reverse the red light stimulation (Table 5).

Light quality experiments indicated the qualitative nature of the light requirement. Data in Table 4 indicated that this response was probably a red/far-red sensitive system. Germination was not stimulated by blue, green, or far-red light but was stimulated by red light. The far-red light was able to reverse the red light stimulation if it was given as the last light treatment (Table 5). This light response was indicative of a phytochrome medicated system.

In another test, seed were placed in continuous light under standard germination conditions, removed at various intervals (0, 12, 24, 36, 48, 60 and 72 hours), and placed in the dark. Other seed were placed under standard germination conditions, then the jars were removed at various intervals (0, 12, 24, 36, 48, 60, and 72 hours), aerated, and returned to the light conditions. These time studies with the light and anaerobic requirement indicated that under the light conditions used, this seed required a minimum of 24 hours of light (continuous 1800 ft-c light) and 60 hours of

Table 3. The comparison of continuous and interrupted red light in the stimulation of germination of seed of H. limosa under standard germination conditions.

Treatment	Percent Germination
Continuous Red Light:	
12 hours	0
24 hours	79
One Hour Dark Interruption Period in Light Induction:	
12-12	83
8-8-8	82

Table 4. The effect of light wavelength on the germination of seed of H. limosa.

Expt.	Percent Germination			
	Blue(440mμ)	Green(540mμ)	Red(660mμ)	Far-red(750mμ)*
1	0	0	94	0
2	0	0	90	0
3	0	0	96	0

* The numbers in parenthesis indicate the wavelength of maximum light transmission of cellophane filters.

of anaerobic conditions for the stimulation of at least 50% germination (Table 6).

An attempt was made to separate the light requirement from the anaerobic requirement. Seeds were placed in light under aerobic conditions for three days and then transferred to the dark under anaerobic conditions for three more days. There was no germination. The incubation periods were extended to five days under both conditions but no germination occurred. The test conditions were reversed (dark/N_2 to light/air) and again no germination was seen (Table 7).

Studies on the Effect of Cracking the Seed Coat

With many seeds having a light or an O_2 requirement, cracking the seed coat will initiate germination. This was also found to be the case for seed of H. limosa. Table 8 showed that opening the trap-door or cracking the seed coat resulted in germination regardless of the light or atmospheric conditions. Because of the small size of the seed, an average of 15% of the embryos were injured enough to cause their death. Opening the trap-door resulted in total germination of the uninjured seed. Cracking the seed coat at the chalazal end or the side resulted in a much lower germination. Embryo injry accounted for a fair amount of the germination reduction but the major reduction was seen to be the size of the crack itself.

Table 5. The effect of red (660mμ) and far-red (750mμ) light on the germination of seed of H. limosa.

Light Sequence*	Percent Germination
R	82
R-Fr	0
R-Fr-R	75
R-Fr-R-Fr	0
R-Fr-R-Fr-R	58

* The letter 'R' means a total of 24 hours of red light, and the letters 'Fr' mean 3 hours of far-red light. The treatments followed each other with no time lapse between treatments. The seed were incubated in the dark after treatment for 3 days.

Table 6. The minimal requirements of light and anaerobic conditions for germination of seed of H. limosa.

Treatment	Hours/treatment	Percent Germination
Continuous Light* (in N_2)	0	0
	12	0
	24	55
	36	79
	48	90
	60	89
	72	96
Anaerobiosis** (in light)	0	0
	12	0
	24	7
	36	12
	48	39
	60	59
	72	86

*The seeds were incubated in anaerobic/dark conditions after light treatment.

**The seeds were incubated in aerobic/light conditions after anaerobic treatment.

Table 7. The effect of separation of the anaerobic and light requirement on the germination of seed of H. limosa.

Experiment	Treatment*		Percent Germination
1	Light/air	Dark/N_2	0
2	Dark/N_2	light/air	0

* The seeds were kept in the first treatment for 3 days, then transferred to the second treatment for 3 days.

Table 8. The effect of cracking the seed coat on the germination of seed of H. limosa in air, light, and dark.

Treatment	Percent Germination*
Trap door opened, light/air	82
" " " , dark/air	84
Side cracked, light/air**	10
" " , dark/air**	5
Chalazal end cracked, light/air	65
" " " , dark/air	55

* Embryo injury during cracking of seed coat resulted in an average 15% decrease in germination. The size of the crack in the seed coat directly influenced germination.

** Seed that did not germinate through the crack would germinate through the trap-door when placed into standard germination conditions (anaerobic).

It was noted that germination from side and chalazal cracks was abnormal. Germination from side cracks resulted in a bow shaped seedling with the radicle and cotyledon ends remaining within the seed. When germination occurred from the chalazal end, the seed coat remained on the radicle resulting in a restriction of root development.

Excised embryos showed no dormancy when placed on a nutrient agar of modified White's medium. The embryos were excised under a safe-light but it was found that light had no effect on the growth of the embryos. The embryos began to grow immediately upon being placed on the agar, unaffected by either light or atmospheric conditions (Table 9). By the second day, the embryos had reached a length of 2-3 mm and root formation was beginning at the radicle. Embryos placed on distilled water agar showed little or no growth but slight elongation was seen in the upper end of the cotyledon. The indications are that the embryos require nourishment from external sources for growth and development.

Studies on the Effect of Temperature on Germination

Temperature studies indicated that this seed had a very narrow temperature tolerance during germination. Germination would not occur above 35° or much below 25°. At 35°, germination was inhibited 90% and delayed for 2

Table 9. The growth of excised embryos on nutrient agar in air, N_2, light, and dark.

Growth Conditions	Growth (mm)*
Sterile dist. water, light/N_2	0.6
Sterile dist. water, light/air	0.6
Nutrient agar, light/air	2.2**
Nutrient agar, light/N_2	2.3**
Nutrient agar, dark/air	3.0
Nutrient agar, dark/N_2	3.1

* The embryos averaged 0.4 mm in length, and growth was measured after two days.
** The growth figures cited included injured embryos which did not grow. If the viable embryos only are averaged, the figures on all treatments approach each other.

Table 10. The effect of germination stimulators on the germination of H. limosa under unfavorable conditions.

Treatment	Conc. (moles)	Percent Germination
Indoleacetic acid, light/air	0.0001	0
" " , dark/N_2	0.0001	0
Gibberellic acid, light/air	0.001	0
" " , dark/N_2	0.001	0
Kinetin, light/air	0.0001	0
" , dark/N_2	0.0001	0
Thiourea, light/air	0.01	0
" , dark/N_2	0.01	0

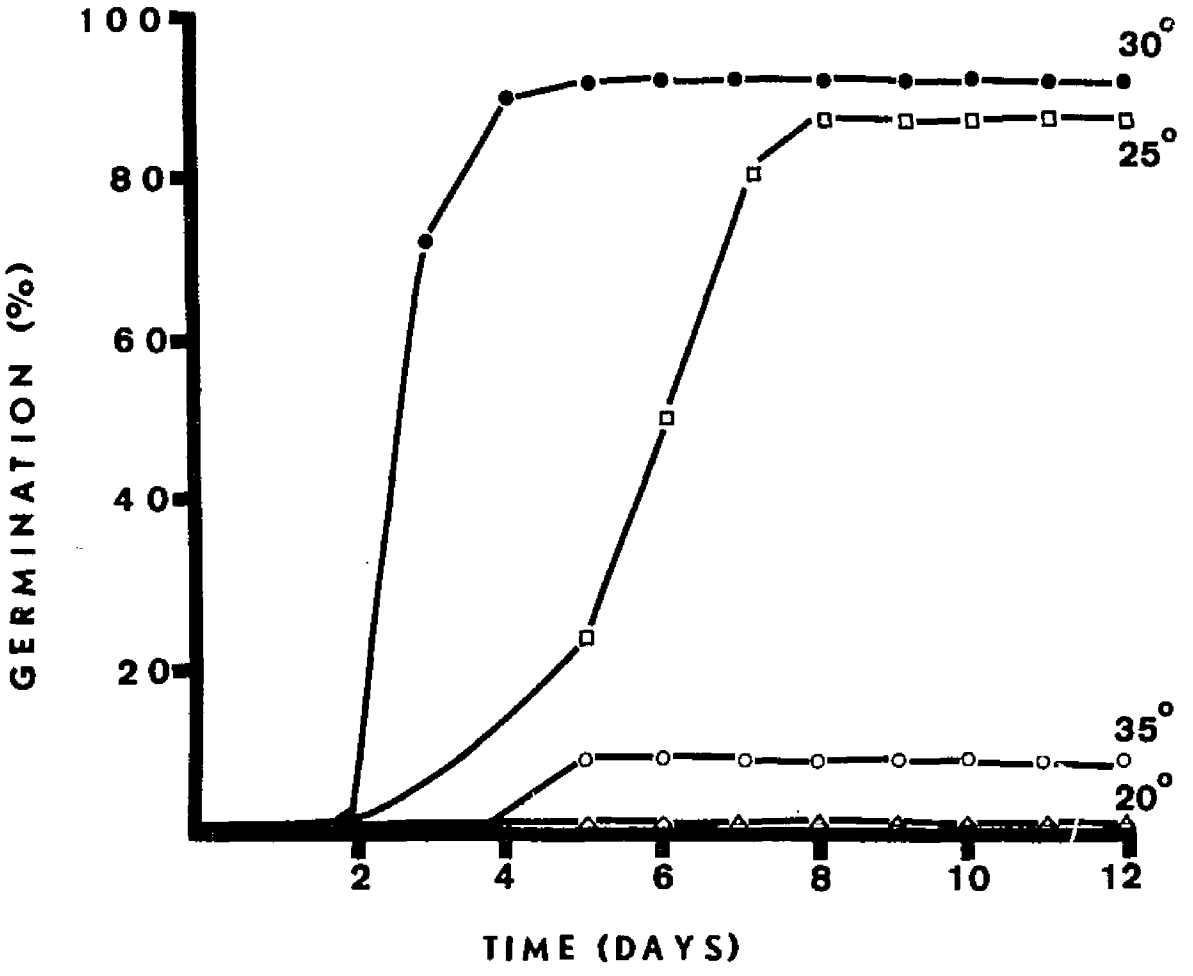
days, while at 20°, germination would not take place at all. Germination was delayed but not inhibited at 25°, and as seen in Figure 4, the optimum temperature for germination was at or near 30°C.

During the temperature study, attempts were made to induce dark or aerobic germination by various temperature treatments. The seeds were frozen (imbibbed and dry) for 24-48 hours and then transferred to air/light or N_2/dark at 30°, but no germination was induced. Cold storage at 5° or in alternating temperatures (15°/30°, 20°/30°) for up to three months had no effect on dormancy in air or dark.

Studies on the Induction of Germination by Various Chemicals

Lettuce and tobacco seed studies have shown that for photoblastic seeds, various compounds would substitute for the red light effect, so an attempt was made to substitute indolacetic acid, kinetin, gibberellic acid, and thiourea for the light or anaerobic requirements. The range of concentrations used were: indolacetic acid - 10^{-6} to 10^{-4}M, gibberellic acid - 10^{-4} to 10^{-2}M, kinetin - 10^{-6} to 10^{-3}M, and thiourea - 10^{-3} to 10^{-1}M. The concentrations reported in the literature as stimulatory are seen in Table 10. The results for all concentrations were the same, no germination induced in the absence of light and anaerobic conditions.

Figure 4. The effect of various constant temperatures on the germination of seed of *H. limosa*.


100
80
60
40
20
GERMINATION (%)
30°
25°
35°
20°
2
4
6
8
10
12
TIME (DAYS)

Respiration Studies

Because of the requirement for anaerobic conditions, it was assumed that the seed contained an active fermentative respiratory system. Dry seeds were weighed and placed on moist filter paper in Warbury vessels. The vessels were flushed for three minutes with N_2 and the CO_2 evolution was measured under standard light and temperature conditions. CO_2 evolution averaged 2.1 ul/ 24 hrs/mg seed during germination, increasing to 4.1 ul/24 hrs/mg seed upon emergence of the radicle (Figure 5). The seed also possessed an active aerobic respiration system. Measurements of O_2 uptake showed that the seeds consumed 2.2 ul O_2/ 24 hrs/mg seed at a steady rate even though the seeds never germinated (Figure 6).

Metabolic inhibitor studies showed that certain substances inhibited respiration and germination very effectively under anaerobic conditions. As seen in Figure 5, iodoacetate at 10^{-3}M stopped respiration, and also blocked germination (Table 11). Other inhibitors (DNP, NaCN, NaN_3, and KH_2AsO_4, all at 10^{-3}M) had from moderate to no effect on fermentative respiration (Figure 7), but as is seen in Table 11, DNP and arsenate showed 85 and 70% inhibition of germination respectively. Azide (N_3^-) had no effect on respiration or germination at 10^{-3}M, but it did prevent the synthesis of chlorophyll in the

Figure 5. The inhibition of anaerobic respiration of seed of H. limosa with 10^{-3}M iodoacetate. The arrow at 30 hours indicates introduction of the inhibitor. The legend is as follows: ● , respiration of uninhibited seed and the change in slope at 72 hours indicates the emergence of the radicle. ▲ , respiration of seed inhibited with iodoacetate.

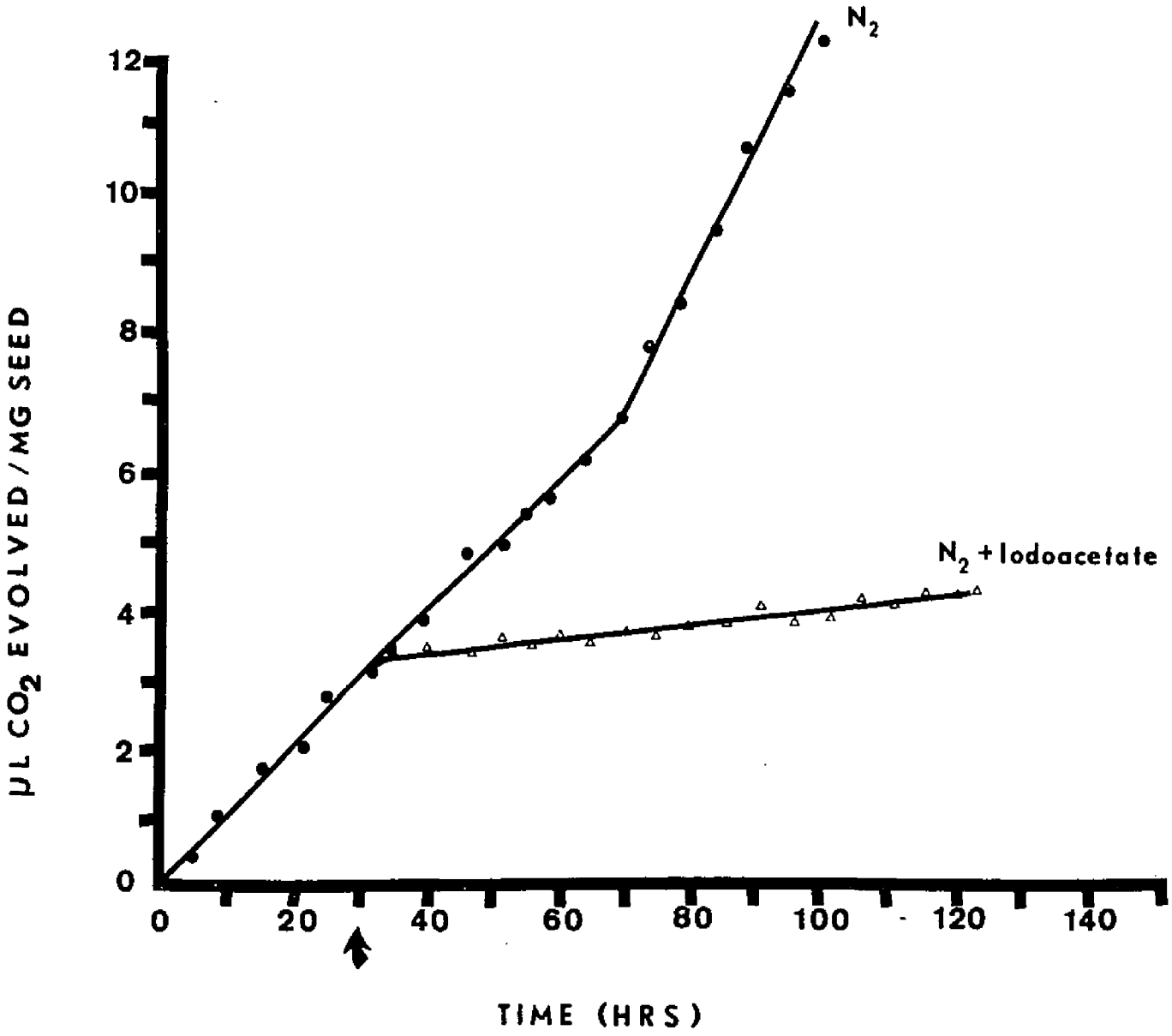

N_2
N_2 + Iodoacetate
12
10
8
6
4
2
0
0
20
40
60
80
100
120
140
µL CO_2 EVOLVED / MG SEED
TIME (HRS)

Figure 6. The uptake of oxygen by seeds in air, under standard light and temperature conditions.

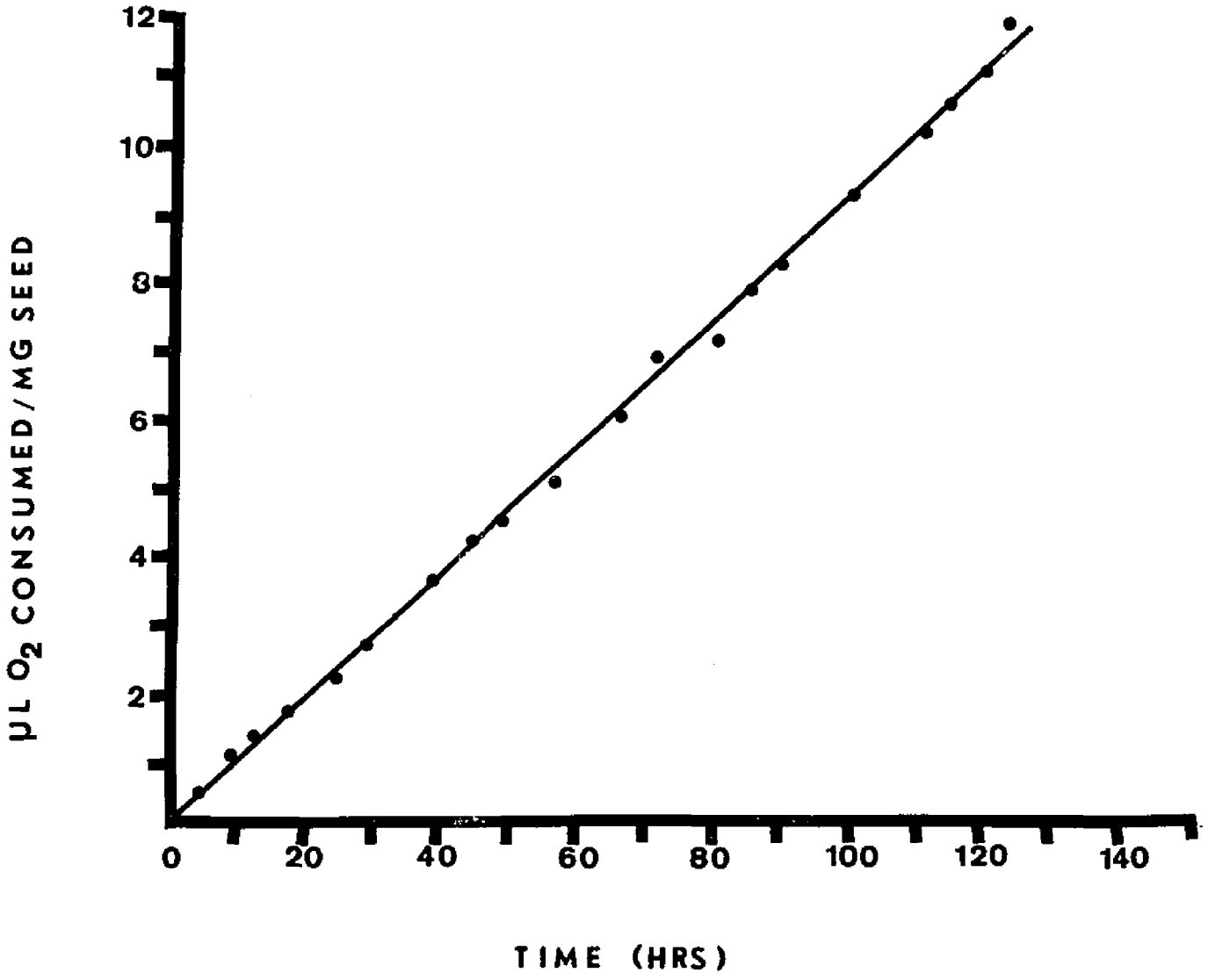

12
10
8
6
4
2
0
20
40
60
80
100
120
140
µL O2 CONSUMED/MG SEED
TIME (HRS)

Table 11. The effect of metabolic inhibitors on the germination of whole and cracked seed.

Treatment	Conc. (moles)	Percent Germination
Whole seed, light/N_2		
NaCN	0.001	91
KH_2AsO_4	0.001	30
Iodoacetate	0.001	0
NaN_3	0.001	89
DNP	0.001	15
Cracked seed, light/air or N_2		
NaCN	0.01	93
Iodoacetate	0.001	96*

* Germination (emergence of the radicle) occurred but the seedling failed to grow more than 2 mm in either air or N_2.

Figure 7. The effect of metabolic inhibitors on the anaerobic respiration of seed of H. limosa under standard germination conditions.

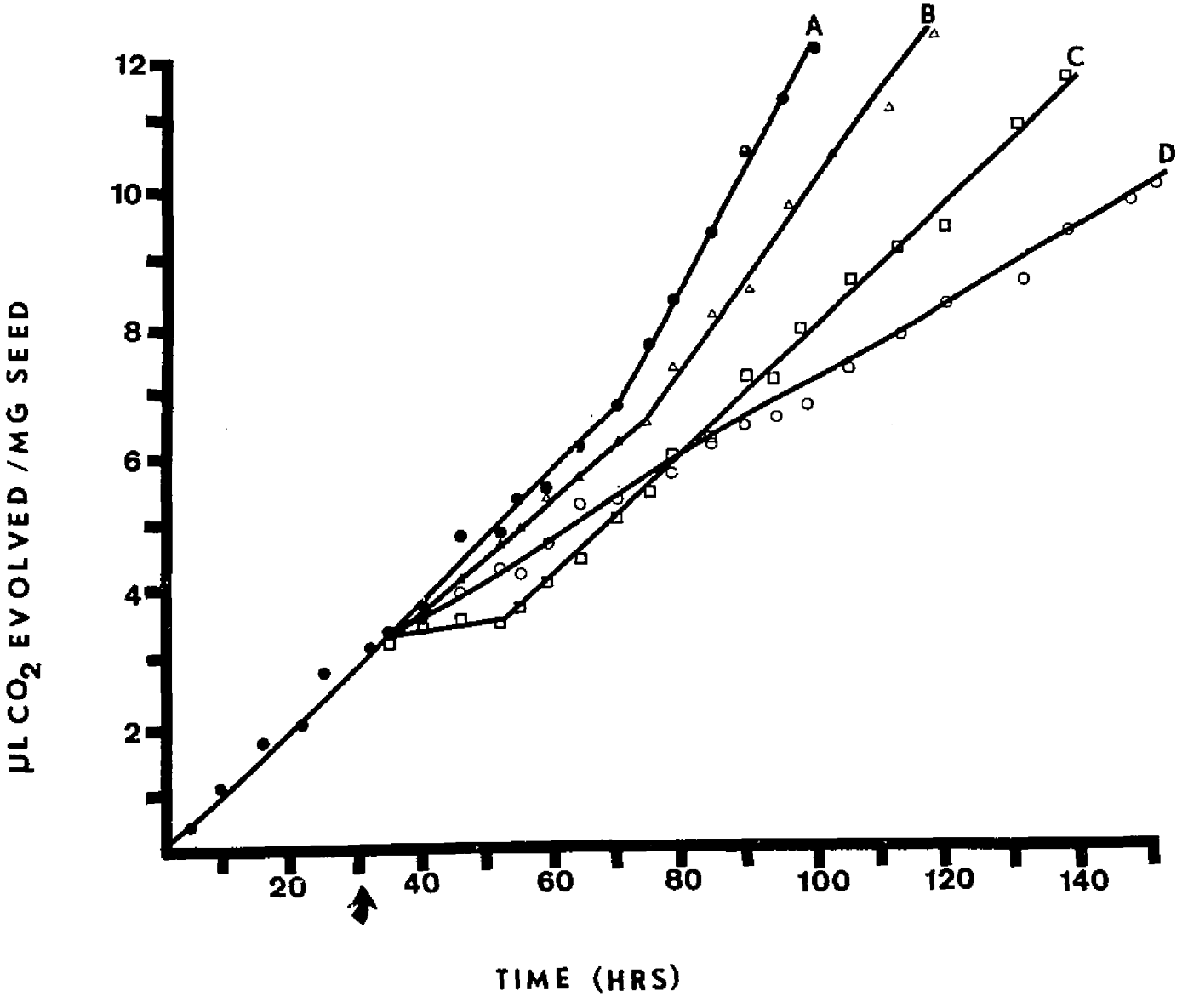
A -uninhibited respiration

B -10^{-3}M NaCN or NaN_3

C -10^{-3}M KH_2AsO_4

D -10^{-3}M DNP

The arrow at 30 hours indicates the introduction of the inhibitor. Germination (change in the slope of the line) is unaffected by NaCN and NaN_3 but it is strongly inhibited by DNP and KH_2AsO_4.


A
B
C
D
12
10
8
6
4
2
20
40
60
80
100
120
140
µL CO2 EVOLVED /MG SEED
TIME (HRS)

seedling. Cyanide had no effect on respiration or germination under anaerobic conditions, but it was seen that under aerobic conditions, there was a stimulation of germination (Table 12). Though this stimulation was not great (about 22%), it led to the idea that since cyanide was also a good reducing agent, perhaps an enzyme or compound required reduction before the germination process could proceed.

The Effect of Reducing Agents on Germination

A variety of reducing agents were tried in an attempt to replace the anaerobic requirement. Table 12 gives the list of compounds tried and some representative concentrations. As can be seen from this table, under full air (21% O_2) there was only an indication of stimulation of germination (13%) with reduced glutathione. However, if the oxygen level was lowered to 10% there was a strong tendency for these reducing agents to stimulate germination. When the oxygen level reached 5%, these reducing agents were very effective in stimulating germination (Table 12). The reducing compounds showed little indication of specificity but reduced glutathione was generally the best stimulator and least toxic of the agents. After germination, considerable inhibition of seedling growth was seen for the higher concentrations of glutathione, mercaptoacetate, and thiosulfate. Dithiothreitol had no

Table 12. The effect of reducing agents on the germination of H. limosa in three concentrations of oxygen, under standard light and temperature conditions.

Treatment	Conc.(moles)	Percent Germination 21% O_2	10% O_2	5% O_2
Distilled water	-	0	0	0
NaCN	0.005	22	-	-
Glutathione (red.)	0.01	13	62	71*
	0.001	0	10	47
Cysteine	0.01	0	5	38*
	0.001	0	0	52
Mercaptoethanol	0.01	0	-	-
	0.001	0	0	22
	0.0001	-	0	26
Mercaptoacetate	0.01	0	-	-
	0.001	0	0	8*
	0.0001	-	0	42
Thiosulfate	0.01	0	0	20*
	0.001	0	0	39*
Dithiothreitol	0.001	0	0	0
	0.0001	0	0	0

* Germination was stimulated but the growth of the seedling was inhibited.

stimulatory action and was found to inhibit germination under standard germination conditions.

Histological Studies

To better understand the processes involved in germination, a histological study of the seed was undertaken. Standard microtechnique was employed in the killing, fixing, and sectioning of the seeds. It should be noted at this stage that this seed is very small (0.7 mm long) and difficult to section. The seed coat warps during sectioning and one is seldom able to obtain photomicrogrpahs with both sides of the seed coat in focus under high power. The seed coat was also very fragile and shattered badly if the microtome blade was not extremely sharp. During this study, it was found that the use of plastic embedding material gave excellent results in preventing the seed coat from shattering (Figure 10).

The seed barrel-shaped with ridges running down its length (Figure 8). The embryo was situated in the center of the seed as a long straight rod (Figure 9). The endosperm consisted of large cells filled with starch grains and there was apparently no oil or protein stored in the endosperm since staining for these components was negative. As seen in Figure 9, the trap-door and chalazal ends were structually distinct in both the seed coat and the embryo. About 4/5 of the embryo was cotyledon with

Figure 8. Whole germinating seed of H. limosa showing the emerging radicle and the trap-door through which it emerged.

Figure 9. Longitudinal section of the seed of H. limosa at 100 X.

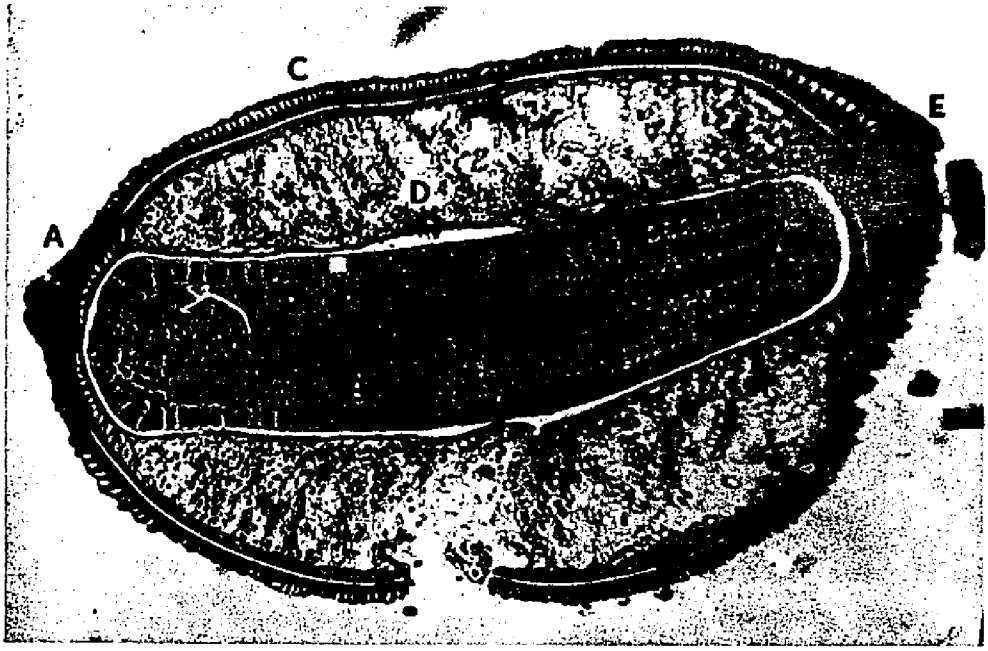
A. Trap-door end of seed

B. Embryo

C. seed coat

D. endosperm and starch grains

E. Chalazal end of seed


C
E
D
A

the remaining 1/5 consisting of the root tip and apical growing point. The cells of the cotyledon, especially those at the chalazal end, contained large numbers of protein bodies--the aleurone grains (Figure 17A). The aleurone grains consisted of a membrance structure enclosing many protein crystals (Figure 17B).

The seed coat was sectioned and stained for suberin, lignin, and secondary cellulose with the following stains; suberin-light green/sudan IV/hemalum; lignin-methylene blue/ruthenium red; secondary cellulose-safranin/fast green. The seed coat consisted of a single layer of thickened cells subtended by two layers of material that did not take any of the stains used (Figure 10D). These two layers were separated by a strip of cellulose. The outer layer of cells had extensive secondary cellulose filling the lower half of each cell (Figure 10B), while the upper half of the cells were very thin and stained positive for suberin and lignin (Figure 10F). Only on the ridges running down the seed coat did the upper part of the cells form a solid structure. In the valleys and on either end of the seed, these thin upper parts were crushed and broken away leaving a serrated-like seed coat (Figure 10C). The outer layer of cells could be divided into two types, the regular seed coat cells and the trap-door seed coat cells (Figure 10A, C). The

trap-door cells differed from the regular seed coat cells only along the inner edge where the individual cells were distinguishable. The cells of the seed coat were not individually distinct (Figure 12). The embryo sac was surrounded by a dead, crushed nucellus (Figure 10E) except at the trap-door end where the nucellus cells were large and living (Figure 10B). These living cells (beneath the trap-door) bordered, but did not extend into the endosperm, and judging from their location, probably had no part in the breakdown of the starch grains.

Sections made at various time periods into germination indicated that there was a progressive breakdown of intercellular material between certain cells of the trap-door. This area of cells is the cleavage line along which the trap-door opens and the arrow of Figure 12 denotes this cleavage line. The cleavage line appeared to occur at the 5th or 6th cell from the base of the pointed tip of the trap-door. Figure 11 showed that there were 5 cells from the edge of the trap-door to the darkened mass of the center of the trap-door. As seen in Figures 12 through 15, and especially clear in Figure 15, this corresponded to the area undergoing intercellular breakdown (indicated by the arrows). Figure 12 is a control or zero time section showing these trap-door cells held tightly together along the cleavage line. At 24 hours

Figure 10. Plastic-embedded section of seed showing the structure of the seed coat.

A. Trap-door cells of the seed coat.

B. Living nucellus cells subtending trap-door cells.

C. Outer layer of cells of the seed coat.

D. Non-staining parts of seed coat.

E. Crushed, dead nucellus cells bordering the endosperm.

F. Seed coat ridge; the upper part of cell stained for suberin and lignin, and was crushed and broken in the valley between the ridges; note the broken upper parts on other side of seed.

G. Lower part of seed coat cell; it stained for secondary cellulose.


A
C
D
E

Figure 11. Dorsal view of a portion of the trap-door. The upper section is the trap-door and the lower piece is the underlying nucellus cells. The dark center of the trap-door is an out-of-focus view of the point of the conical shaped trap-door. See Figure 16 for the lateral view.

Figure 12. The trap-door of H. limosa at zero time showing the closely packed cells of the seed coat. The arrow indicates the trap-door cleavage line. Note the difference in the cells of the trap-door as compared to the cells of the seed coat toward the margin of the figure.

into germination, there was still little apparent breakdown of material between these cells (Figure 13). At 48 hours into germination, there was evidence of breakdown of material between the two cells along the cleavage line (Figure 14). By 72 hours, the breakdown was extensive and significant weakening of the seed coat had occurred (Figure 15). Between 72 and 84 hours the radicle forced open the trap-door and germination was completed (Figure 16).

The force required for the opening of the weakened trap-door was probably provided by the expansion of aleurone grain filled cells of the embryo. The chalazal half of the embryo contained large cells packed with these aleurone grains (Figures 17 and 18). During germination these grains were seen to dissolve, releasing what probably was osmotically active material. Figure 18A showed the grains as they began to dissolve in the cotyledonary cells of the embryo late in the incubation period. As the cells elongated, the aleurone grains completely disappeared (Figure 18B).

The dissolving of the protein crystals in isolated grains was followed in glycerine with dark field phase microscopy. The first noticable change, which occurred after two hours of water uptake, was a clearing or decrease in refractive index inside the aleurone grains.

Figure 13. The trap-door of H. limosa after 24 hours incubation under germination conditions. The arrow indicates the cleavage line of the trap-door. There is little change in the intercellular material at this time.

Figure 14. The trap-door of H. limosa after 48 hours incubation under germination condition. The arrows indicate the cleavage line. The lower arrow shows the beginning of breakdown of intercellular material.

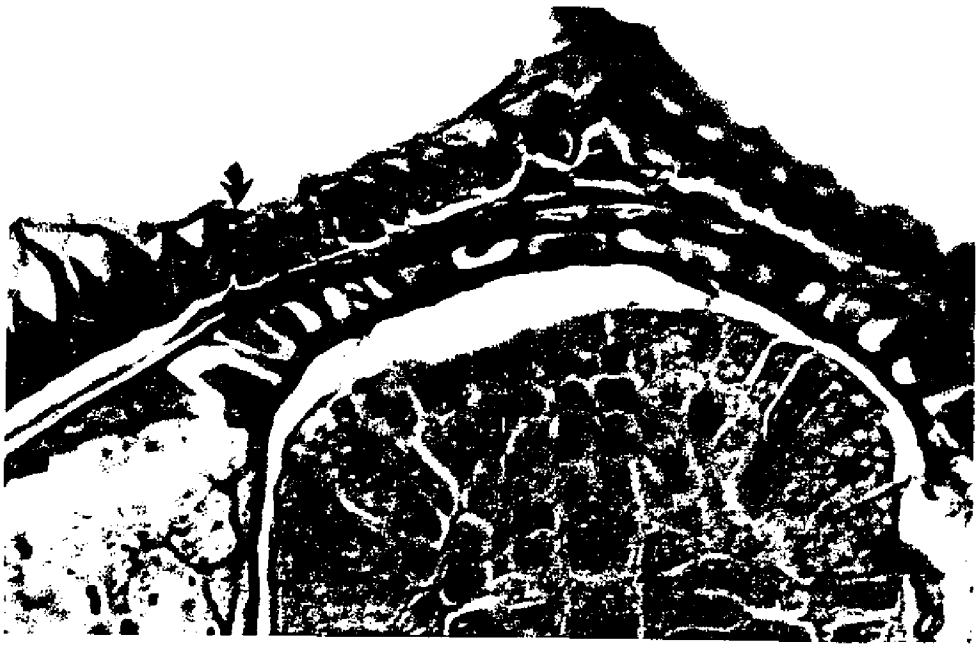


Figure 15. The trap-door of H. limosa at about 72 hours of incubation. Note the breakdown of intercellular material at the trap-door cleavage line (arrow).

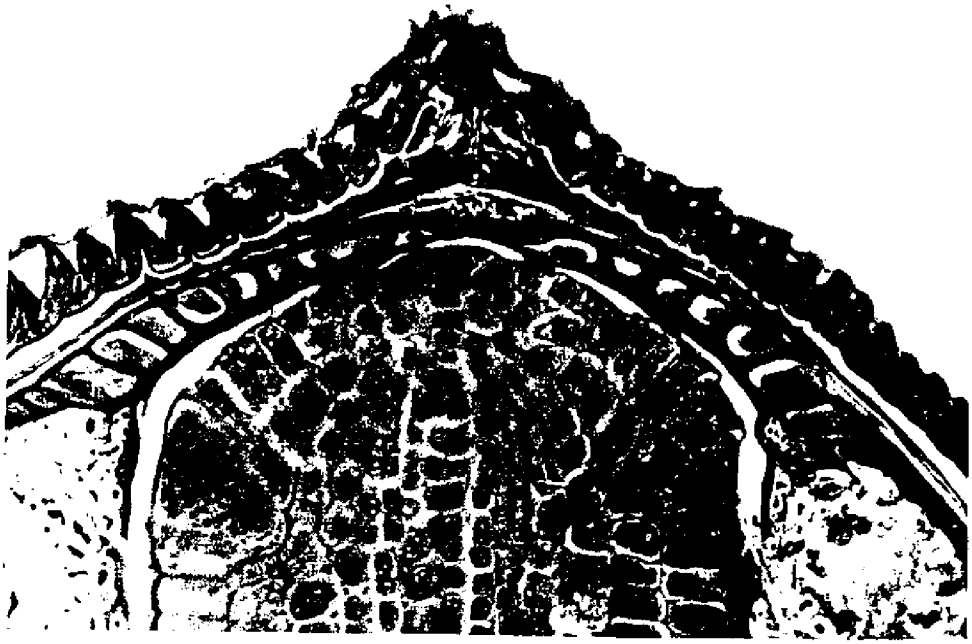
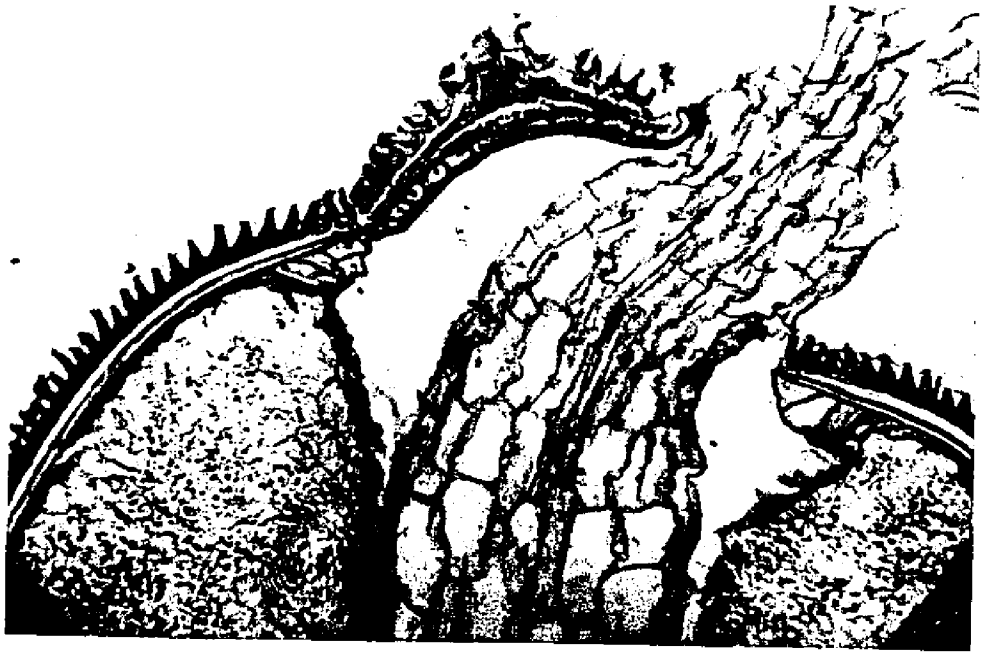


Figure 16. The trap-door of H. limosa at 72-84 hours of incubation. The radicle has pushed open the weakened trap-door and emerged.



The change from the crystalline to the liquid state took 10 hours for completion. The refractive index of this liquid was slightly different from the glycerine and it appeared to pass through the membrane of the grain into the glycerine. After 24 hours, the glycerine had replaced the aleurone fluid and only the outline of the membrane remained.

It is noteworthy that this embryo can be roughly divided into two parts. The radicle end of the embryo (Figure 19B) underwent cell division but not a marked elongation; while the chalazal end (Figure 19A) underwent only an extensive elongation with no cell division. Cell elongation began before the trap-door opened, with cell division being delayed until after the radicle had emerged. Observation of the radicle showed that these cells did not undergo division until about 8 hours after the radicle had emerged.

The embryo of the cattail, Typha latifolia, emerged through a trap-door similar to that seen for the seed of H. limosa (Figure 20). To compare the germination requirements of Typha to those of Heteranthera, Typha seed were harvested from the marsh near Hammond, Louisiana, and placed in a variety of germination conditions. The results in Table 13 showed that Typha had no requirement for anaerobic conditions and, in fact, the seed were

Figure 17. The aleurone grains of H. limosa.

A. The chalazal end of the embryo showing the numerous aleurone grains inside the cells of the embryo.

B. An oil emersion view (970 X) of the aleurone grains showing the protein crystals within the membrane of the grains. The cytoplasm had shrunk away from the cell wall during fixing.

A

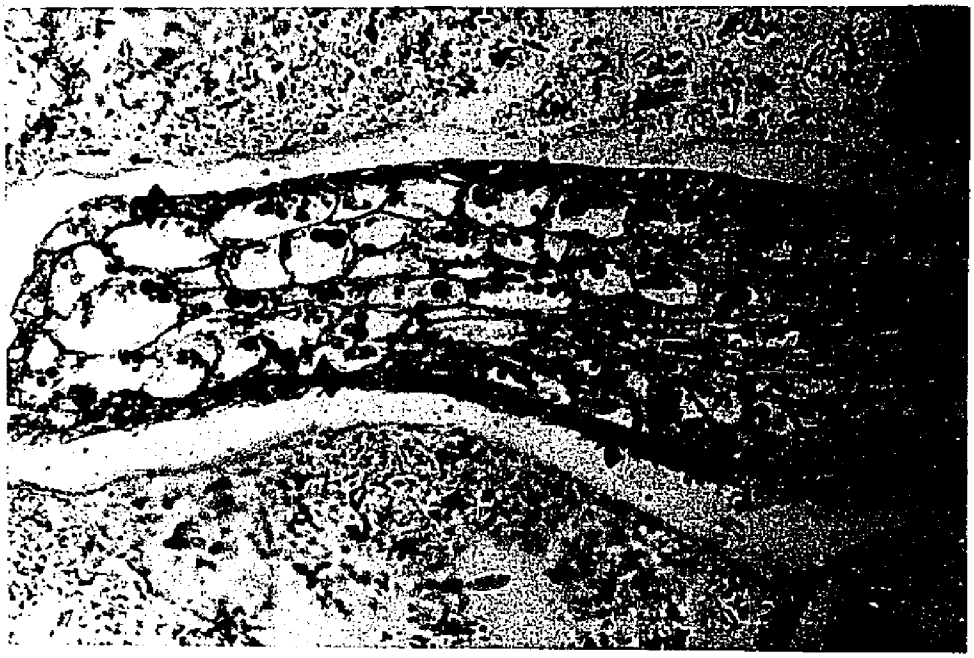
B

Figure 18. The chalazal end of the embryo of H. limosa showing the disappearance of the aleurone grains.

A. View of aleurone grains at the start of elongation of the chalazal end of the embryo. This is about 72 hours into germination and trap-door has not yet opened. The arrows indicate aleurone grains with the protein crystals dissolved.

B. View of embryo about 12 hours after the trap-door has opened. The arrows indicate cells in various stages of elongation. Note the lack of aleurone grains.

A



B

Figure 19. Embryo of H. limosa about 12 hours after germination. The embryo is divided roughly into two sections:

A. the chalazal end that undergoes elongation but no cell division.

B. the radicle end that undergoes cell division but no extensive cell elongation.

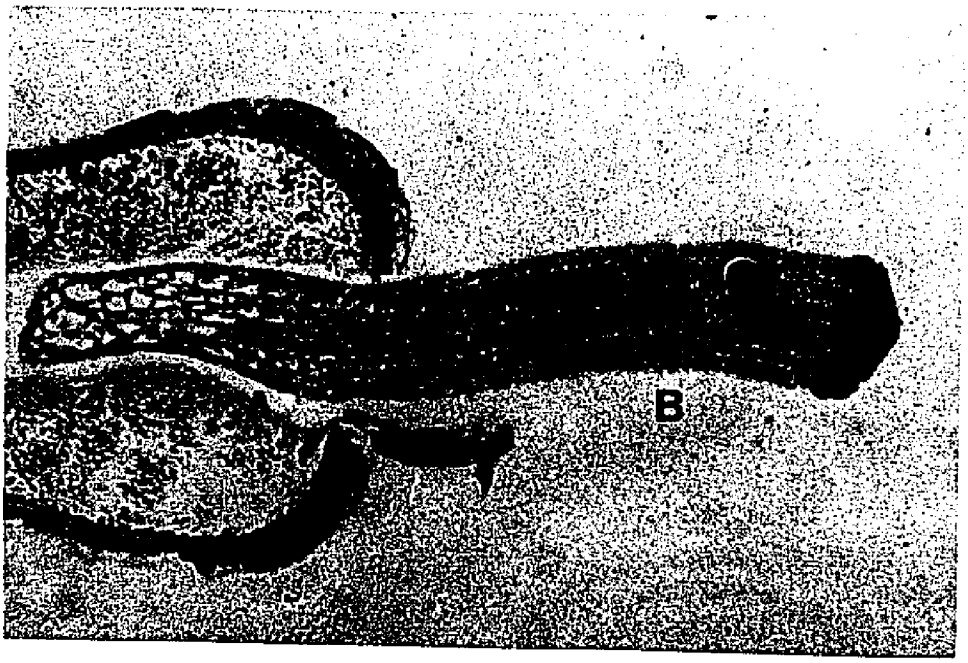

B

Figure 20. Germinating seed of Typha latifolia in air showing the emergence of the radicle and the trap-door (arrow).

Table 13. The germination of Typha latifolia (cattail) under various germination conditions.

Treatment*	Percent Germination
Light/anaerobic	0
Light/aerobic	75
Dark/anaerobic	0
Dark/aerobic	0

* The incubation temperature and light conditions were those used for the germination of H. limosa. Incubation time was 5 days for all treatments.

inhibited by anaerobiosis. The seed did show a definite requirement for light during germination.

DISCUSSION

Reports of the positive effect of oxygen on seed germination are wide spread in the literature. It is postulated that sometimes a hard seed coat blocks or at least interferes with the exchange of gases, or sometimes inhibitors in the seed increase the seed's oxygen requirement beyond that received through the seed coat. Under these conditions, increasing the oxygen tension will often overcome the dormancy and germination is able to proceed (7, 18, 52, 81, 105). At the other end of the oxygen versus germination spectrum are those seeds capable of germinating at, or near, anaerobic conditions. The requirement for anaerobiosis during germination, however, is limited to a very few seeds. Morinaga's (62, 63) survey of the influence of oxygen on the germination of 75 seeds revealed that only two (Typha, Cynodon) were enhanced by near-anaerobic conditions. Since that survey in 1926, only a few other seeds (Peltrandra, Pinus, Alisma) have been shown to be enhanced by low oxygen tensions during germination (53, 58, 86, 88). To this somewhat exclusive group of seeds, the seed of Heteranthera limosa is to be added.

Sifton (88) studied in detail the germination of one of the anaerobic seed, the cattail, Typha. Unfortunately, this seed did not show an anaerobic requirement in all lots tested.

For those lots requiring low oxygen tensions, maximum germination occurred at about 2% oxygen so the seed preferred near-anaerobic rather than anaerobic conditions. Typha seeds taken from the marsh south of Hammond, Louisiana, exhibited no near-anaerobic requirement and were inhibited from germinating under low oxygen conditions (Figure 20 and Table 13).

The seed of H. limosa, compared to Typha, had a definite requirement for anaerobic conditions. The data of Figure 3 showed that these seeds were inhibited by oxygen concentrations about 0.5%. Though capable of germinating under zero oxygen, the seedlings did not develop (Table 1). This has been seen for all seeds capable of anaerobic germination. Upon emergence of the radicle, the metabolism of these seeds appear to shift to an oxygen (if only in trace amounts) requiring system.

Coupled with the anaerobic requirement, H. limosa had a requirement for light during germination. This light response was both quantitative and qualitative in nature. Table 2 showed that light intensity and duration had a direct influence on germination rate, but not on whether the seed would germinate. Though the evidence was not conclusive, indications were that the quantity of light (intensity times time) was more

important than the intensity of the light alone. It was seen that 80 hours of high intensity (1800 ft-c) light had the same influence on germination as 240 hours of low intensity (600 ft-c) light (Table 2).

The rather prolonged light treatment required was not unique to seed germination. Toole et al (99) reported the seed of Paulownia required 48 hours of light to stimulate germination. Time studies with continuous white or red light showed that H. limosa seed required 24 hours for stimulation. The light could be given all at once or in increments totaling 24 hours. The only effect was a change in the germination time, so it appeared that the product of red light action was stable and accumulated until a critical concentration was reached for germination. It should be noted that light stimulation was accomplished only under anaerobic conditions and, as seen from Table 7, seeds put in light and oxygen (air) and then transferred to N_2 and dark are not stimulated to germinate. Therefore, the product of light induction was either broken down, inactive, or not produced in the presence of oxygen.

In attempting to compare the phytochrome mediated light response of this seed to those reported in the literature, it became increasingly apparent that the effect of anaerobiosis on light sensitive seed varied

with the species studied. Erograstis were stimulated by anaerobiosis only when given during the inductive dark period. Anaerobic conditions during the light period, however, severely inhibited germination (27). Certain varieties of tobacco were reported to be rendered insensitive to light induction when given anaerobic conditions during the imbibition period (74). Lettuce seed did not appear to be affected by anaerobic conditions during imbibition and light induction, but anaerobiosis during the post-induction period blocked the seed's escape from far-red inhibition (41). Thus the fact that the action of the phytochrome mediated system of H. limosa was not affected by low oxygen tension and may even be inhibited by oxygen could not be considered unique, but it was certainly different from the response reported for aerobic seed.

Sifton (88) noted that those Typha seed that appeared 'aerobic' were able to germinate in the dark after treatment with alternating temperatures, but those Typha seed requiring anaerobiosis were not stimulated to germinate by this treatment. Likewise, the light requirement of H. limosa seed could not be replaced by this temperature treatment.

The various chemical compounds reported in the literature to have a simulatory action on the germination

of aerobic seed, were unable to replace the light requirement of H. limosa seed. As can be seen in Table 10, these compounds had no stimulatory action in air or in the dark. The light requirement appeared absolute, and at no time did this seed respond to any treatment and germinate in the dark. It was difficult, therefore, to draw analogies to the results seen here and those reported for the many light-snesitive aerobic seed.

One condition (other than standard germination conditions) that allowed germination was the physical cracking of the seed coat. In many oxygen starved seed, cracking the seed coat stimulated germination in air. This effect was often ascribed to an increase in oxygen supply for the internal tissues (9, 28, 50, 56). The stimulation of germination seen in Table 8 cannot be explained in this light. Oxygen in this case was normally inhibitory so the cracking of the seed coat must affect germination in a different manner. A series of observations seem to rule out the leaching or breakdown of an internal inhibitor: 1) the seeds would not germinate in air after long periods of soaking in distilled water, 2) the effect of oxygen in other seeds was to destroy inhibitors while in this case, oxygen inhibited germination of the seed, 3) the size and position of the crack in the seed coat had a direct influence on the rate of germination, and

4) the embryo would grow and develop immediately after being removed from the seed and placed on nutrient agar in dark/air conditions.

In certain seed, the seed coat appeared to take up water and exchange gases readily but the seed coat restricted germination by acting as a physical barrier to germination (7). This type of dormancy has been seen for Rubus, Capsicum, Phaseolus, Eucalyptus, and lettuce to mention a few (3, 4, 7, 39). As in the case of these other seeds, mere picking of the seed coat of H. limosa to expose the endosperm was not sufficient to allow germination. The side cracked seed germinated only when the crack extended more than half-way around the seed. This mere exposure of the endosperm through the cracked seed coat resulted in only 10-15% germination (Table 8). Cracking the chalazal end was as effective as opening the trap-door only when the crack nearly removed the end of the seed. Again insufficient cracking resulted in lower germination rates (Table 8). The seed appeared incapable of generating sufficient force to split open the seed coat when the crack was small. There was no normal (through the trap-door) germination in air when the crack was not large enough to allow germination. Germination proceeded normally in insufficiently cracked seed when placed in N_2 so the key to germination appeared

to be the opening of the trap-door.

The data from metabolic inhibitor studies indicated that this seed obtained its energy through fermentative respiration. The two aerobic inhibitors, cyanide and azide, had no effect on respiration or anaerobic germination, while the fermentative inhibitor, iodoacetate, completely blocked respiration and germination. The effect of DNP and arsenate on respiration was not as dramatic as iodoacetate but germination was greatly inhibited (Figures 5, 7, and Table 11). This strong inhibition by DNP under anaerobic conditions was also seen for rice; so it was apparent that DNP acted on levels other than oxidative phosphorylation, i.e. substrate phosphorylation (87).

This seed either had a nonfunctional cytochrome system in air, or it was extremely resistant to cyanide or azide poisoning. Seeds placed in 10^{-2}M cyanide germinated and the seedling appeared to develop normally under the standard (anaerobic) germination conditions. The seeds were germinated in air (trap-door opened manually) in 10^{-2}M cyanide with no inhibition of germination or seedling development. Seeds treated in the same manner using iodoacetate as the inhibitor were completely inhibited in N_2 and in air. It is entirely possible that the seeds were able to meet all their energy requirements

both in air and in N_2 through the fermentative pathway, and for aquatic plants this may be the rule instead of the exception. Rice has been shown to possess a very active fermentative system, capable of sustaining a large amount of growth under anaerobic conditions (96, 104).

The seeds, however, were capable of utilizing oxygen, and manometric studies (Figure 6) showed a steady uptake of oxygen in air; so it was apparent that there was a nonfermentative respiratory system present and functional in these seeds. The possibility of a non-cytochrome electron-transport system operating in these seeds could not be ruled out. Poljakoff-Mayber and Evenari (72) noted that in lettuce seed, substrate oxidation could possibly occur through pathways other than the TCA cycle and the cytochrome system. They suggested an ascorbic acid oxidase system as such a possibility.

The reducing conditions required for germination were satisfied by a number of compounds (Table 12). These compounds had varying degrees of effectiveness, but in general there was no specificity seen. Reduced glutathione appeared to be the most effective and least toxic. Certain concentrations of various of these compounds proved rather toxic to the developing seedlings and in the case of dithiothreitol, this reducing agent was ineffective in stimulating germination and very toxic when placed on seedlings.

The effectiveness of these reducing agents was directly related to the oxygen concentration. Under the influence of these reducing compounds the seeds were able to tolerate and germinate in oxygen tensions ten times that which was normally inhibitory.

The action of the various reducing agents led to the hypothesis that reducing conditions, rather than the absence of oxygen, were the key to the germination of these seeds. Indirectly, evidence appeared to indicate that an enzyme or compound required reduction before the germinative processes could go to completion. Various workers (73, 78, 79, 84) had noted the beneficial effects of reducing agents and conditions on the germination of seed. Certain enzymes had been shown to be activated or released by these reducing conditions. Rowsell and Goad (78) reported the release of β-amylase from barley endosperm after reducing agent treatment.

One of the proposed roles of gibberellin in seed germination was the release or activation of enzymes from endosperm tissues. Paleg (70), Varner (103), Simpson and Naylor (89), and others (13, 14, 15, 39, 42, 66, 80) have reported an increase in enzymatic activity upon treatment of seeds with gibberellin. There is good evidence that amylase was released by the action of gibberellin. However, with seed of H. limosa, there was no germination

in air upon treatment with gibberellin. The negative results seen with H. limosa seed did not mean that gibberellin was not active within the seed. From the present knowledge of gibberellin's action, it was possible that the enzymes required for germination were being released, but without the proper reducing environment, these enzymes were inactive.

The results reported in Figures 12 through 16 supported the contention that there was an enzymatic breakdown of intercellular material along the cleavage line of the trap-door. This breakdown appeared to weaken the trap-door sufficiently to allow the embryo to push it open and complete germination. Breaking open the seed coat by the embryo could be accomplished by either embryo cell division, cell elongation or both. In lettuce, corn, barley, and certain beans, cell elongation provided the force necessary for germination, with cell division occurring only after the radicle has emerged (30). Observation of cell division in the embryo H. limosa showed that cell division did not begin until several hours after the radicle had emerged. Thus the force behind the opening of the trap-door was provided by the osmotically mediated elongation of certain cells of the embryo (Figure 19A). This osmotically mediated elongation was also seen for certain other seeds. Scheibe and Lang (80) reported that for lettuce,

the osmotic uptake of water regulated expansion of the embryo, and they believed that this expansion was phytochrome controlled. Chen and Thimann (15) hypothesized that the germination of Phacelia was mediated through the light influenced osmotic uptake of water. This was also the conclusion drawn for the force required for the germination of Typha (88).

Sifton (88), observed and photographed the release of osmotically active material from isolated aleurone grains. From his description of the embryo of Typha, it was noted that H. limosa had nearly the same anatomical makeup. Sifton's reported histological studies of the germination of Typha gave no indication of any seed coat breakdown, and he did not believe that any other action besides the expansive force of the embryo was required for the germination of Typha. Unfortunately, a detailed histological study of the seed coat was not undertaken to determine whether there was a breakdown of material along the trap-door. The morphological changes during water uptake by the isolated aleurone grains and the processes of cotyledon elongation of H. limosa were identical to those described by Sifton for Typha; so the processes of germination are very similar, if not the same, for both Typha and Heteranthera.

SUMMARY

1) The seeds of the aquatic plant Heteranthera limosa were seen to have two primary requirements for germination: anaerobic conditions and light. Dormancy was due to the seed coat which acted as a mechanical barrier to germination. This mechanical restraint to germination was overcome only if certain environmental conditions were provided. These were: a) white light (or red light in the region of 660 mμ), and b) anaerobiosis or reducing conditions.

2) The light requirement appeared to be a phytochrome mediated action because red light (660 mμ) stimulated germination and far-red light (750 mμ) reversed this stimulation. This phytochrome system is unique in that it was unaffected by anaerobic conditions. The light response was not photoperiodic; rather the germination rate appeared to be controlled by a duration times intensity factor (total amount of radiation). Under the experimental conditions employed, at least 24 hours of light was required for stimulation of 50% germination.

3) The seed appeared to obtain most of the energy required for germination through a fermentative pathway. Germination and fermentative respiration were

unaffected by either cyanide or azide but were completely blocked by iodoacetate. Arsenate and DNP had a moderately inhibitory effect on fermentative respiration, while greatly inhibiting germination. The seedlings would develop in N_2 or air in the presence of 10^{-2}M cyanide. Azide did not affect germination or seedling development, but it completely blocked chlorophyll synthesis.

4) The seeds required an exposure of about 60 hours to anaerobic conditions for germination. Reducing agents could substitute for anaerobiosis and stimulated germination if the oxygen concentration was below 10%. Reduced glutathione, cysteine, thiosulfate, mercaptoethanol, and mercaptoacetate were capable of stimulating germination. Some of these compounds were toxic to seedling development at higher concentrations. Dithiothereitol was inhibitory to both germination and seedling development in N_2 and air.

5) The mechanical restraint of the seed coat was apparently overcome by the enzymatic breakdown of intercellular material along a cleavage line of the trap-door. Sections of seed made at various times during germination showed an evidence of a progressive breakdown of material along this line which significantly weakened the seed coat. The embryo pushed open the trap-door by the

expansive force of certain cells of the cotyledon. This expansive force was provided by the apparent release of oxmotically active material from the aleurone grains which filled the cells of the cotyledon. The processes regulating the release of this material are unknown. It was hypothesized that the enzymatic breakdown of trap-door material was mediated by the reducing conditions. The role of light in germination was not known but it appeared coupled to the reducing requirement. Possibly light functioned in the release of enzymes while the reducing conditions provided the proper environment for their activity.

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VITA

James Earl Marler was born in Cleveland, Ohio, December 20, 1939; son of Otis Earl Marler and Dorothy Lemmon Marler. He attended public schools in Corpus Christi, Texas and graduated from W. B. Ray High School in 1958. He entered the University of Miami, Coral Gables, Florida, and received the Bachelor of Science degree in biology in May, 1962.

He was employed as a research assistant at the University of Texas' Institute of Marine Science in Port Aransas, Texas, for one year. In June 1963, he entered the Graduate School of the University of Texas and in the summer of 1965 received a Master of Arts degree in marine microbiology. During this time, he coauthored three publications with his major professor, Dr. Chase Van Baalen.

In the fall of 1965, he accepted a teaching position at Del Mar College at Corpus Christi. He entered the Graduate School of Louisiana State University in 1966 for advanced study in plant physiology in the Department of Botany and Plant Pathology, and is now a candidate for the degree of Doctor of Philosophy in Botany.

He is married to the former Harriet Maurine Smitherman of Corpus Christi, Texas and has two children; Yvette Maurine, age 7 and Annette Michelle, age 4.

LIST OF ERRORS

Page	Line	Should Read
11	24	enzymes
33	23	injury
71	8	light-sensitive
90	No. 100	Tuan, D. V.
91	No. 105	*Xanthium*

EXAMINATION AND THESIS REPORT

Candidate: James Earl Marler

Major Field: Botany

Title of Thesis: A Study of the Germination Process of Seeds of Heteranthera limosa

Approved:

John B. Baker
Major Professor and Chairman

R D Anderson
Dean of the Graduate School

EXAMINING COMMITTEE:

Virginia R. Williams

James G. Gosselink

Charles A. Lieberman

M. John P. Chilton

Date of Examination:

May 1, 1969